Bioactive Diterpenes from the Fruits of *Detarium microcarpum*

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A fruit pulp extract of *Detarium microcarpum* showed inhibition of the growth of the plant pathogenic fungus *Cladosporium cucumerinum* and of the enzyme acetylcholinesterase, implicated in Alzheimer's disease. Fractionation of this extract led to the isolation of four new clerodane diterpenes, 3,4-epoxyclerodan-13*E*-en-15-oic acid (**1**), 5α,8α-(2-oxokolavenic acid) (**2**), 3,4-dihydroxyclerodan-13*E*-en-15-oic acid (**4**), and 3,4-dihydroxyclerodan-13*Z*-en-15-oic acid (**5**). Also isolated were 2-oxokolavenic acid (**3**) and copalic acid (**6**). Structure elucidation of the compounds was carried out by spectroscopic data interpretation and by the X-ray crystallography of **2** and **4**. Three of the new clerodane diterpenes (**1**, **2**, and **5**) showed both antifungal activity and inhibition of acetylcholinesterase, and **3** showed a slight inhibition of this enzyme.

The genus *Detarium* (Caesalpiniaceae) is represented in West Africa by three species. One of these, *D. microcarpum* Guill. & Perr.,[1] which occurs in forests and the dry savannah, is of interest because of its use in African traditional medicine. In particular, the pulp of the fruit is used to treat dizziness, meningitis, and stomach diseases, and it is employed in numerous magical treatments. These fruits are also commonly eaten as food in the whole of West Africa and are known to be very rich in vitamin C.[2−4] Previous studies of the bark led in particular to the isolation of two tetranorditerpenes, two clerodane diterpenes (2-oxokolavenic acid and *cis*-2-oxokolavenic acid), and copalic acid, a labdane diterpene.[5,6] Moreover, extracts of the bark showed moderate antibacterial and molluscicidal activity.[7−9] 2-Oxokolavenic acid, also isolated from the leaves, as well as other clerodane diterpenes, exhibited insect antifeedant activity.[10] Clerodane diterpenes are also known to inhibit fungal growth.[11]

In an ongoing search for bioactive compounds from medicinal plants, a CH_2Cl_2 extract of the pulp of the fruit of *D. microcarpum* showed antifungal properties against the plant pathogenic fungus *Cladosporium cucumerinum* and inhibition of the enzyme acetylcholinesterase. The isolation, structure elucidation, and biological activities of four new clerodane diterpenes (**1**, **2**, **4**, and **5**), a known clerodane diterpene (**3**), and a known labdane diterpene (**6**) from this extract are reported herein.

Results and Discussion

Initial analysis of the CH_2Cl_2 extract of *D. microcarpum* fruit pulp was performed by LC/UV/MS^n techniques, which showed the major constituents to be diterpenes: typical fragments of diterpenes could be detected among the major constituents.[12] A bioassay-guided fractionation of this extract was undertaken by countercurrent chromatography (CCC), medium-pressure liquid chromatography (MPLC), and open column chromatography on silica gel, leading to the isolation of four new clerodane diterpenes and two known diterpenes.

The molecular formula of compound **1** was determined as $C_{20}H_{32}O_3$ by HRESIMS (pseudomolecular ion at *m*/*z* 343.22384 [M + Na]$^+$). This implied the presence of five unsaturated bonds and/or ring structures. The ^{13}C NMR data (DEPT) (Table 1) showed the presence of 20 carbon signals represented by five methyls, six methylenes, four methines, and five quaternary carbon atoms. The ^{13}C NMR spectroscopic data revealed the presence of a carboxyl function (δ_C 170.9, C-15) and a trisubstituted double bond (δ_C 114.3, C-14 and δ_C 164.7, C-13). Analysis of the gHMBC spectrum showed that the vinylic proton (δ_H 5.70, H-14) (Table 2) correlated with the carboxylic carbon (δ_C 170.9, C-15), which indicated that there is a conjugated system (C-15, C-14, and C-13). Further, the methylene protons H-11 (δ_H 1.28) and H-12 (δ_H 2.28) showed coupling with C-13 (δ_C 164.7), suggesting that C-11, C-12, and C-13 are adjacent. The gCOSY spectrum indicated linear coupling between protons H-14 (δ_H 5.70) and H-16 (δ_H 2.18), confirming that C-16 (δ_C 19.6) is linked to the quaternary carbon, C-13 (δ_C

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Table 1. ^{13}C NMR Chemical Shifts (δ) of Compounds **1**, **2**, and **4**−**6** (125 MHz, $CDCl_3$)

position	1	2	4[a]	5	6
1	20.0	35.7	19.3	18.3	39.1
2	25.1	198.8	29.8	25.8	19.4
3	62.6	128.7	77.0	81.8	42.1
4	65.9	169.4	78.1	75.8	33.6
5	38.1	39.8	43.7	41.9	55.5
6	30.5	30.1	30.2	28.3	24.5
7	27.2	26.9	29.2	27.7	38.3
8	37.0	35.6	38.2	36.9	148.3
9	38.5	39.3	40.2	39.1	56.2
10	41.6	47.2	43.5	42.6	39.7
11	34.1	36.7	35.8	34.4	21.5
12	35.7	34.8	36.8	36.1	40.1
13	164.7	164.4	163.3	164.5	164.3
14	114.3	115.2	116.3	114.5	114.6
15	170.9	171.8	170.5	171.0	171.8
16	19.6	19.5	19.3	19.5	19.2
17	15.6	14.6	16.4	15.8	106.4
18	19.1	21.1	21.9	21.5	33.6
19	21.9	31.2	21.8	20.7	21.7
20	29.6	22.9	29.9	29.2	14.5

[a] The ^{13}C NMR spectrum of compound **4** was run in CD_3OD.

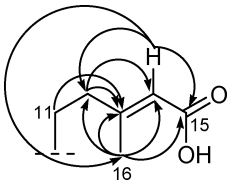

Figure 1. HMBC long-range correlations in the 4-carboxy-3-methylbutyl chain of **1**.

164.7). The long-range $^1H-^{13}C$ couplings shown in Figure 1 suggested that compound **1** contains a 4-carboxy-3-methylbutyl chain. At this stage, four methyls, four methylenes, three methines, three quaternary carbon atoms, and three centers of unsaturation remained to be placed on the diterpene molecule. The C-20 methyl protons (δ_H 0.92) were correlated strongly in the gHMBC spectrum to two methine carbons (δ_C 37.0, C-8 and δ_C 41.6, C-10). Moreover, the methylene protons H-7 (δ_H 1.37 and 1.52) showed a long-range correlation with the C-17 methyl carbon (δ_C 15.6). The $^1H-^{13}C$

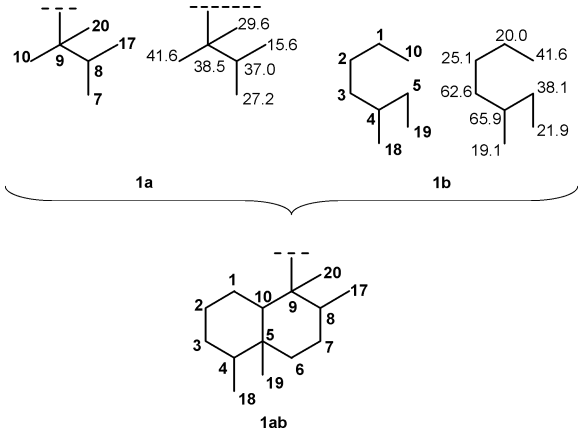

Figure 2. Some structural fragments of **1**.

NMR long-range correlations between the methyl protons appearing at δ_H 0.80 (H-17) and the carbon signal at δ_C 38.5 (C-9) indicated the substructure **1a** (Figure 2). Similarly, substructure **1b** (Figure 2) was generated from the $^1H-^{13}C$ NMR long-range correlations. There were correlations observed between the H-2 methylene protons (δ_H 1.69 and 2.00) and the C-10 methine carbon (δ_C 41.6) and between the H-3 methine proton (δ_H 3.00) and the C-1 (δ_C 20.0) and C-2 (δ_C 25.1) methylene carbons. In turn, the C-18 methyl protons (δ_H 1.18) correlated with the C-5 quaternary carbon (δ_C 38.1) and the C-3 methine carbon (δ_C 62.6). Finally, signals of protons resonating at δ_H 1.29 (H-19) correlated with carbon signals at δ_C 65.9 (C-4) and δ_C 38.1 (C-5). This information, in addition to the $^1H-^{13}C$ long-range correlation between the methyl protons H-19 (δ_H 1.29) and the methine carbon C-10 (δ_C 41.6) suggested a combination of **1a** and **1b** to give a bicyclic perhydronaphthalene derivative **1ab** (Figure 2). The shift to low fields, observed for the signals of C-3 and C-4, indicated that these carbons are linked to an oxygen as an epoxide. Finally, the chain was linked to the C-9 quaternary carbon (δ_C 38.5), which was confirmed by the gHMBC spectrum, showing a strong correlation between the H-11 methylene protons and the C-8 methine carbon. No NOE enhancements were observed between the H-16 methyl protons (δ_H 2.18) and H-14 (δ_H 5.70) (Figure 3), which indicated that this double bond has an *E* configuration. In a $^1H-^1H$ NOE experiment, the H-17 protons

Table 2. ^1H NMR Data (*J* in Hz) of Compounds **1**, **2**, and **4**−**6** (500 MHz, $CDCl_3$)

position	1	2	4[a]	5	6
1	1.30 m	2.56 d (18.56)	1.42 m	1.54 m	1.00 m
	1.40 m	2.82 dd (6.35, 18.55)	1.83 m	1.63 m	1.73 m
2	1.69 m		1.59 m	1.76 m	1.50 m
	2.00 m		2.01 m	2.12 m	1.57 m
3	3.00 d (5.80)	5.80 s	3.48 s	4.90 s	1.17 m
					1.39 m
5					1.08 m
6	1.36 m	1.48 m	1.08 m	1.14 m	1.30 m
	1.63 m	1.84 m	2.31 m	2.00 m	1.55 m
7	1.37 m	1.30 m	1.32 m	1.40 m	1.96 m
	1.52 m	1.60 m	1.47 m	1.45 m	2.38 m
8	1.50 m	1.60 m	1.67 m	1.65 m	
9					1.57 m
10	1.38 m	1.42 m	1.74 m	1.72 m	
11	1.28 m	1.18 m	1.28 m	1.32 m	1.50 m
	1.90 m	1.68 m	1.96 m	1.93 m	1.67 m
12	1.98 m	2.06 m	2.06 m	2.29 m	1.98 m
	2.28 m	2.06 m	2.37 m	2.06 m	2.31 m
14	5.70 s	5.65 s	5.67 s	5.70 s	5.67 s
16	2.18 s	2.14 d (0.98)	2.14 s	2.20 s	2.16 s
17	0.80 d (6.40)	0.95 d (7.32)	0.82 d (6.83)	0.83 d (6.80)	4.49 s
					4.85 s
18	1.18 s	1.94 d (1.46)	1.20 s	1.18 s	0.86 s
19	1.29 s	1.24 s	1.20 s	1.23 s	0.80 s
20	0.92 s	0.82 s	0.97 s	0.96 s	0.68 s

[a] The ^1H NMR spectrum of compound **4** was run in CD_3OD.

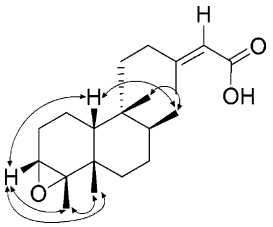

Figure 3. Main NOESY correlations observed for **1**.

(δ_H 0.80) showed correlations with the H-20 (δ_H 0.92) and proton H-10 (δ_H 1.38), which indicated that the C-20 methyl group is in an equatorial position, the C-17 methyl group is axial, and H-10 and the side chain are also axial. Moreover, a NOE was recorded between H-3 (δ_H 3.00) and H-10 (δ_H 1.38) and between H-3 and H-19, and revealed that rings A and B have a *cis*-junction. A NOE between H-3 and the H-18 methyl protons (δ_H 1.18) and between the latter and H-19 (δ_H 1.29) finally revealed the relative stereochemistry of **1**. Thus, compound **1** was assigned as a new clerodane diterpene derivative, 3,4-epoxyclerodan-13*E*-en-15-oic acid.

Compound **2** showed a pseudomolecular ion at *m*/*z* 341.20890 $[M + Na]^+$ in the HRESIMS, corresponding to the molecular formula $C_{20}H_{30}O_3Na$. Compared to compound **1**, most of the chemical shift values for the carbons and protons of compound **2** were similar (Tables 1 and 2), which suggested that **2** is also a clerodane diterpene. As signals for the H-1 protons (δ_H 2.56 and 2.82) had shifted to a quite low field, this suggested the presence of an adjacent carbon with a low electron density. The gHMBC spectrum implied that these protons had strong correlations with a carbonyl carbon (δ_C 198.8, C-2), shown from the gHSQC and DEPT NMR spectra to be ketonic. The chemical shifts of C-3 and C-4 (δ_C 128.7 and 169.4, respectively) indicated that they are linked by a double bond instead of an epoxide. Consequently, gHSQC and DEPT NMR spectroscopic analysis showed that C-3 is a methine carbon linked to a low-field proton (H-3, δ_H 5.80) and that C-4 is a quaternary carbon. $^1H-^{13}C$ long-range NMR correlations of the C-18 methyl protons (δ_H 1.94) with C-3 and C-2 suggested that C-18 is linked to C-4. Thus, **2** is 2-oxocleroda-3,13*E*-dien-15-oic acid, also called 2-oxokolavenic acid, a compound already isolated from several plants, including the bark and leaves of *D. microcarpum*.[5,10] However, the stereochemistry of **2** is new, as demonstrated by X-ray analysis (Figure 4). The molecule consists of a *cis*-decalin moiety with an extended 3-methyl-pent-2-enoic acid side chain. The bond lengths and angles in **2** are normal for such a compound. In the crystal structure of **2**, symmetry-related molecules are linked by an O−H···O hydrogen bond, involving the acid proton on atom O-1 and the carbonyl oxygen O-3. In this manner, a helical polymer-type structure is formed, extending in the *b* direction as shown in Figure 4b. Compared to 2-oxokolavenic acid already isolated from the bark and leaves of *D. microcarpum*,[5,10] compound **2** possesses a *cis*-decalin and a *trans* orientation of C-8 and C-9. Thus, **2** was assigned as 5α,8α(2-oxokolavenic acid).

Compound **3** could be identified as 2-oxokolavenic acid, according to the comparison of data furnished by multidimensional NMR spectroscopy experiments and EIMS measurements and those of the literature.[13] As in the same compound found in the bark and leaves of *D. microcarpum*,[5,10] **3** possesses a *trans*-decalin and a *cis* orientation of C-8 and C-9.

Compound **4** showed a pseudomolecular ion at *m*/*z* 361.23507 $[M + Na]^+$ in the HRESIMS, corresponding to the molecular formula $C_{20}H_{34}O_4Na$. Even though the NMR data of **4** were obtained in CD_3OD, the majority of its ^{13}C and ^1H NMR data were similar to those of compound **1** (see Tables 1 and 2). The only difference from compound **1** was the opening of the epoxide ring, leading to the formation of a diol at C-3 and C-4. Thus, signals of these carbons were shifted to lower field: δ_C 77.0 (C-3) and δ_C 78.1 (C-4). This compound was consequently established as 3,4-dihydroxyclerodan-13*E*-en-15-oic acid. The relative configuration of **4**, confirmed by X-ray crystallographic analysis (Figure 5), is different from that of the isomer previously isolated from *Relhania genistifolia* L. (Asteraceae).[14] The molecule consists of a *cis*-decalin moiety with an extended 3-methylpent-2-enoic acid side chain. The bond lengths and angles in **4** are normal for such a compound. In the crystal structure of **4**, symmetry-related molecules are linked by three O−H···H hydrogen bonds to form a trimeric-type structure. This extends to form a 2D sheet in the *bc* plane as shown in Figure 5b.

Compound **5** gave a pseudomolecular ion at *m*/*z* 361 $[M + Na]^+$ in the ESIMS. Analysis of its 1D and 2D NMR data (see Tables 1 and 2) showed **5** to be an isomer of **4**. A long-range $^1H-^1H$ NMR experiment showed that the vinylic proton H-14 (δ_H 5.70) had a strong correlation with the methyl protons H-16 (δ_H 2.20), which indicated that the double bond has a *Z* configuration. Concerning the decalin substructure, the strong NOE observed between the C-20 methyl protons (δ_H 0.96) and H-17 (δ_H 0.83) indicated that this methyl group is in an axial position and the one at C-17 is in an equatorial position, and the side chain is equatorial. As H-10 (δ_H

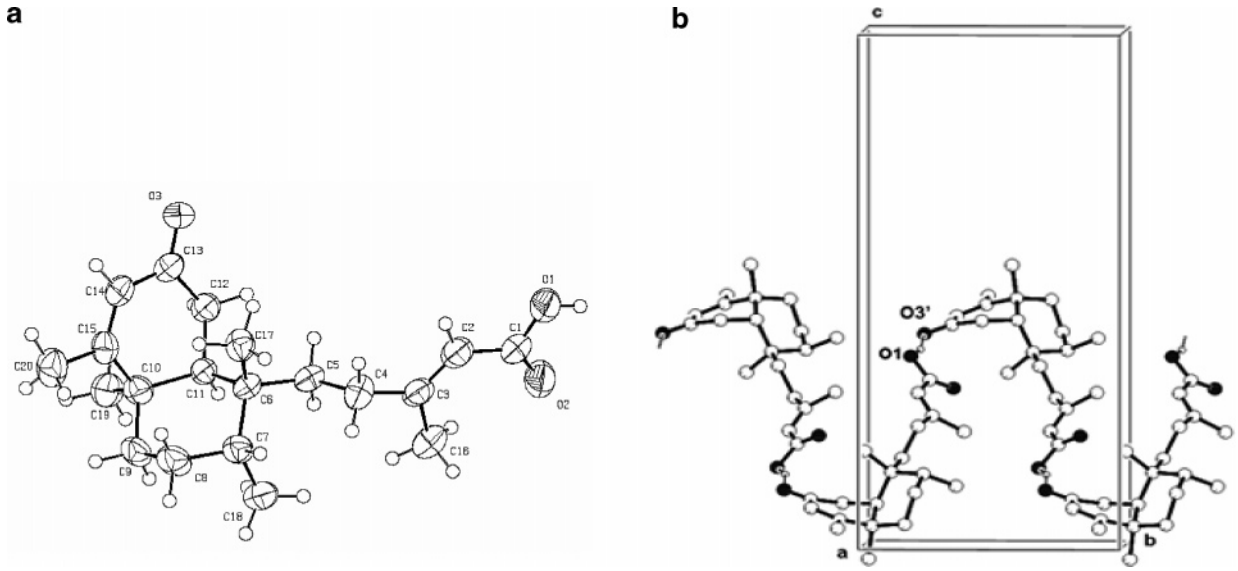

Figure 4. (a) View of the molecular structure of **2**, showing the crystallographic numbering scheme and thermal ellipsoids at the 50% probability level. (b) Crystal packing of **2** viewed down the *a* axis. The hydrogen bonds are shown as dashed lines.

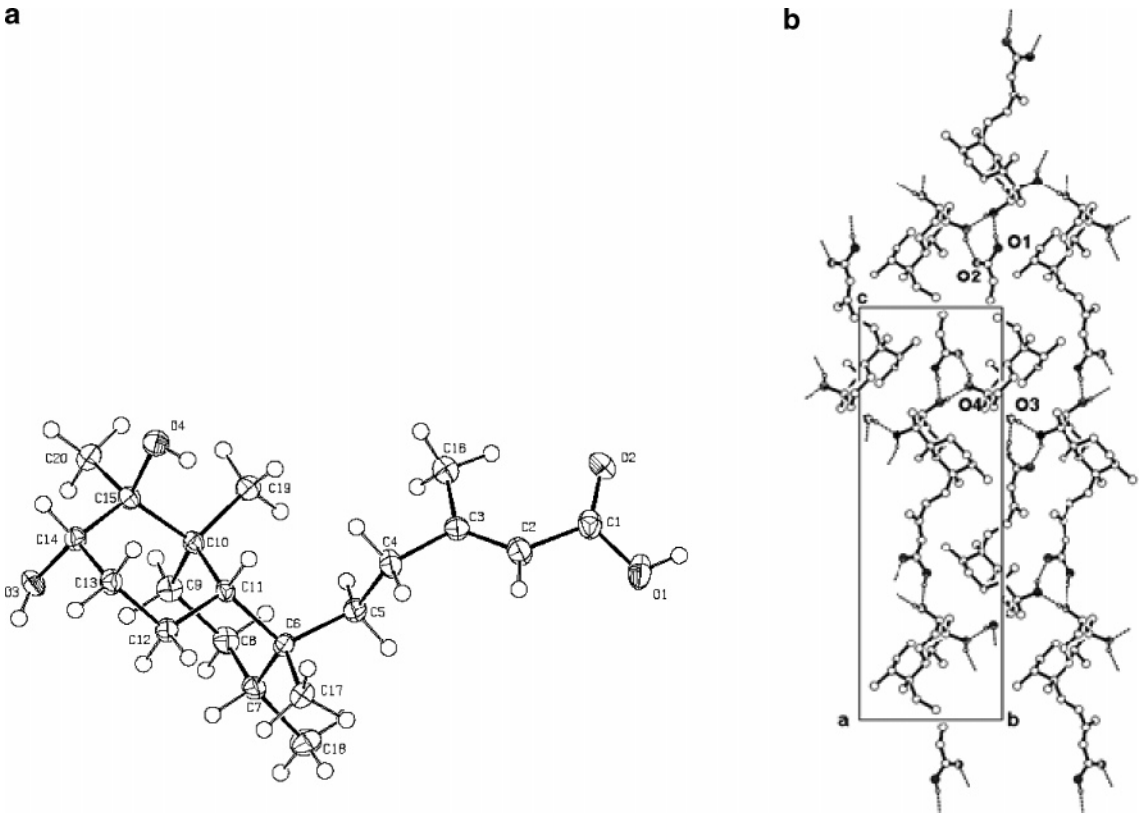

Figure 5. (a) View of the molecular structure of **4**, showing the crystallographic numbering scheme and thermal ellipsoids at the 50% probability level. (b) Crystal packing of **4** viewed down the *a* axis. The hydrogen bonds are shown as dashed lines.

1.72) did not correlate with H-20, this suggested that H-10 is also in an axial position. The H-10 proton showed a NOE correlation with H-19 (δ_H 1.23), implying that rings A and B have a *cis*-junction. As H-10 did not correlate with H-18 (δ_H 1.18), it was inferred that the hydroxyl group on C-4 is equatorial. That the hydroxyl group on C-3 is also equatorial was proved by the strong correlation between H-3 (δ_H 4.90) and H-18. Thus, compound **5** is a new isomer, 3,4-dihydroxyclerodan-13*Z*-en-15-oic acid.

In the HRESIMS, compound **6** showed a pseudomolecular ion at *m*/*z* 327.22953 $[M + Na]^+$, corresponding to the molecular formula $C_{20}H_{32}O_2Na$. Analysis of its ^{13}C and ^1H NMR data (Tables 1 and 2) showed compound **6** to be copalic acid, which has already been isolated from the bark of *D. microcarpum*.[6] However, 1D and 2D NMR data have not been assigned completely and details of the determination of the relative configuration have not been reported. Thus, a spectroscopic study was performed in the present investigation. Concerning the 4-carboxy-3-methylbutyl chain, the absence of a long-range $^1H-^1H$ correlation between H-14 (δ_H 5.67) and H-16 (δ_H 2.16) suggested that the double bond is in the *E* configuration. A NOE between H-20 (δ_H 0.68) and H-11 (δ_H 1.67) showed that the methyl group (C-20) has an axial orientation and the side chain is equatorial. The long-range coupling between H-20 and the C-19 methyl protons (δ_H 0.80) indicated that this methyl group (C-19) has an equatorial position, implying that the C-18 methyl group is axial. As H_3-18 (δ_H 0.86) showed a NOE with H-5 (δ_H 1.08), the presence of a *trans*-decalin ring was shown. This was confirmed by the absence of coupling between H-5 and H-20. Thus, the relative configuration of the labdane diterpene **6** (copalic acid) was demonstrated for the first time.

The pure compounds obtained by bioassay-guided fractionation were tested for inhibition of the growth of *C. cucumerinum* and their effects on acetylcholinesterase (AChE), and the results are presented in Table 3. Compound **2** slightly inhibited the growth of *C. cucumerinum* at 100 μg, while compounds **1** and **5** were moderate inhibitors at 10 μg. Compound **2** proved to be a strong inhibitor of AChE at 0.1 μg. Its isomer, **3**, showed a slight inhibition

Table 3. Bioactivities of Isolated Compounds from *Detarium microcarpum* in TLC-Based Assays

compound	*Cladosporium cucumerium*[a]	acetylcholin-esterase[a]
1	10	10
2	100	0.1
3	>100	1
4	>100	>10
5	10	10
6	>100	>10
miconazole[b]	1	
galanthamine[b]		0.01

[a] Amounts given, in μg, are the minimum inhibitory quantities applied on the TLC plates. [b] Reference compound.

at 1 μg, while compounds **1** and **5** also showed a weak activity at 10 μg. In comparison, the alkaloid galanthamine, used clinically for the treatment of Alzheimer's disease, inhibits the enzyme at 0.01 μg.[15] At the present time, the use of inhibitors of acetylcholinesterase seems to be one of the most efficient approaches to treat the symptoms of Alzheimer's disease. However, most inhibitors of AChE are alkaloids and present some disadvantages, such as a short half-life or side effects.[16] For this reason, it is important to continue the search for new inhibitors, and this class of nonalkaloidal metabolite, clerodane diterpenes, should be investigated in more detail.

Furthermore, this is the first time that clerodane diterpenes have been reported as inhibitors of AChE and as inhibitors of *C. cucumerinum*. It is interesting to note that **5** both affects the growth of *C. cucumerinum* and inhibits AChE, contrary to its isomer **4**, which, like **6**, was inactive. In comparison with its isomer **2**, compound **3** had no effect on the growth of the fungus at 100 μg.

Experimental Section

General Experimental Procedures. Melting points were measured on a Mettler-FP-80/82 hot-stage apparatus and are uncorrected. Optical rotations were determined using a Perkin-Elmer 241 polarimeter (MeOH

or $CHCl_3$, *c* in g/100 mL). UV spectra were measured on an HP 1100 DAD spectrophotometer (Agilent) in MeOH or MeCN, between 190 and 500 nm. ^1H and ^{13}C NMR spectra were recorded on a Varian Unity Inova-500 NMR spectrometer (500 and 125 MHz, respectively) in $CDCl_3$ or CD_3OD; chemical shifts are in ppm as δ values relative to Me_4Si (internal standard). HRESIMS were recorded on a Bruker FTMS 4.7 T instrument, using the positive mode. EIMS were obtained on a Finnigan MAT TSQ-700 triple-stage quadrupole instrument, recorded at 70 eV. ESIMS was performed on a LCQ ion trap (Finningan MAT, San Jose, CA), using the following conditions: negative mode; capillary voltage 2.8 kV; cone voltage 30 V; nebulizing gas (N_2); flow 500 L/h; desolvatation temperature 150 °C; source temperature 120 °C. For LC/MS analysis of the plant extract, a LCQ ion trap (Finningan MAT, San Jose, CA) with APCI interface was used with the following conditions: capillary temperature 150 °C; vaporizer temperature 400 °C; negative mode; sheath gas flow 60 arb, corona needle current 5 μA; spectra (150−900 mu). TLC employed precoated silica gel plates 60 F_{254}, with aluminum-backed sheets (Merck), with detection at 254 nm and with the vanillin-sulfuric acid reagent.[17] Countercurrent chromatography (CCC) was accomplished with a CCC-1000 apparatus (Pharma-Tech) at 1000 rpm, equipped with an SSI LLC 300 pump, 3.0 mL/min, a Knauer 100 mV detector at 254 nm, and a W+W 600 recorder, 100 mV. MPLC separation was done using a Buchi 681 pump equipped with a Knauer UV detector (210 nm) using a C_{18} column (15−25 μm, 40 × 500 mm, Lichroprep, Merck). Open column chromatography was performed using silica gel 60 (40−63 and 70−200 μm, Merck). Analytical HPLC was carried out on a HP 1100 (Agilent) system equipped with a photodiode array detector. The initial extract, fractions, and pure compounds were analyzed on a Nova-Pak RP-18 column (5 μm, 150 × 3.9 mm i.d., Waters). The flow rate was 1 mL/min, and the gradient used was 30% to 100% MeCN in water in 30 min; the UV traces were measured at 210 and 254 nm, and UV spectra (DAD) were recorded between 190 and 500 nm. X-ray crystallographic data collection was carried out with an Oxford Diffraction KM4/Sapphire CCD diffractometer.

Plant Material. Fruits of *Detarium microcarpum* Guill. & Perr. were obtained at Bamako Market (Mali, March 2000). They were identified by D. Diallo, and a voucher specimen of the plant is deposited at the Herbarium of the Laboratory of Pharmacognosy and Phytochemistry, Geneva, Switzerland (No. 2000083).

Extraction and Isolation. The combined pulp and pericarp of the dried fruits (632 g) were extracted with CH_2Cl_2 (1.5 L, 3 × 24 h) and concentrated under vacuum to give 8.8 g of extract. This was then subjected to CCC using hexane−AcOEt−MeOH−H_2O (10:5:5:1) as solvent system. The lower aqueous phase was used as stationary phase, and the upper phase was pumped from the head of the column to the tail. This separation afforded eight fractions (A−H, 240 mL each). Fraction D (1452 mg) was purified by CCC (isooctane−*tert*-butylmethyl ether−MeOH−H_2O, 4:4:3:2) to give seven fractions (DK−DQ, 100 mL each). Fraction DO (330 mg) was purified by crystallization (AcOEt−hexane) and yielded compound **2** (110 mg). Compound **3** (13 mg) was purified by precipitation from fraction DP by the upper phase of the CCC solvent system (isooctane−*tert*-butylmethyl ether−MeOH−H_2O, 4:4:3:2). Fraction G (449 mg) was purified by CCC (hexane−AcOEt−MeOH−H_2O, 1:1:1:1), using the upper phase as mobile phase. Eight fractions were obtained (GK−GR, 120 mL each). Fraction GM (28 mg) was then purified by crystallization (AcOEt−hexane) and yielded compound **4** (16 mg). Fraction C (716 mg) was also purified by CCC using the upper phase of the system hexane−AcOEt−*tert*-butylmethyl ether−MeOH−MeCN−H_2O (4:2:1:3:2:2) as mobile phase. This separation afforded six fractions (CK−CP, 40 mL each). A 158 mg aliquot of fraction CM was then purified by CCC using heptane−CH_3CN−EtOH−H_2O (30:5:20:1) as the solvent system. Two pure compounds were obtained: **1** (9 mg) and **5** (19 mg). Fraction B (1550 mg) was purified by CCC with an upper phase of heptane−CH_3CN−EtOH (6:1:3) as the mobile phase. Seven fractions were obtained (BK−BQ, 100 mL each). Fraction BK (530 mg) was purified by MPLC with MeCN−H_2O (40:60) giving five fractions (BK1−BK5, 70 mL each). Fraction BK4 (141 mg) was separated by open column chromatography on silica gel, with a step gradient of hexane−AcOEt (2:1 to 0:1), affording six fractions (BK4a−BK4f, 40 mL each). Fraction BK4b (55 mg) was purified by precipitation from MeCN, which led to the isolation of compound **6** (5 mg). The purity of the isolated compounds was determined by HPLC/UV analysis. These diterpenes were found to be unstable to heat, light, and acid.

3,4-Epoxyclerodan-13*E*-en-15-oic acid ([5-(5,6-epoxy-1,2,4a,5-tetramethyldecahydronaphthalen-1-yl)-3-methylpent-2-enoic acid] (1): yellow liquid; t_R 16.5 min; $[\alpha]_D^{23}$ +70.6 (*c* 0.1, $CHCl_3$); UV (MeCN) λ_{max} (log ϵ) 228 (4.01) nm; ^{13}C and ^1H NMR data, see Tables 1 and 2, respectively; ESIMS *m/z* 320 $[M - H]^+$ (47), 318 (100), 296 (16), 215 (8); HRESIMS *m/z* 343.22384 $[M + Na]^+$ ($C_{20}H_{32}O_3Na$ requires 343.22492).

5α,8α(2-Oxokolavenic acid) [5α,8α(2-oxocleroda-3,13*E*-dien-15-oic acid)] (2): white needles (AcOEt−hexane); mp 159−164 °C; t_R 11.2 min; $[\alpha]_D^{23}$ −55.1 (*c* 1.2, $CHCl_3$); UV (MeCN) λ_{max} (log ϵ) 238 (4.23) nm; ^{13}C and ^1H NMR data, see Tables 1 and 2, respectively; EIMS *m/z* 319 $[M]^+$ (7), 272 (5), 205 (16), 163 (28), 135 (40), 124 (100), 122 (57), 95 (58), 69 (32), 55 (35); HRESIMS *m/z* 341.20890 $[M + Na]^+$ ($C_{20}H_{30}O_3Na$ requires 341.20926).

2-Oxokolavenic acid [2-oxocleroda-3,13*E*-dien-15-oic acid] (3): white amorphous powder (see ref 13).

3,4-Dihydroxyclerodan-13*E*-en-15-oic acid (4): white needles (AcOEt−hexane); mp 216−221 °C; t_R 7.0 min; $[\alpha]_D^{23}$ −20.4 (*c* 0.9, MeOH); UV (MeOH) λ_{max} (log ϵ) 225 (3.63) nm; ^{13}C and ^1H NMR data, see Tables 1 and 2, respectively; EIMS *m/z* 338 $[M]^+$ (0.2), 320 (43), 303 (3), 277 (22), 225 (100), 207 (42), 189 (46), 163 (38), 149 (18), 137 (34), 123 (44), 113 (16), 109 (34), 95 (79), 84 (36), 69 (29), 55 (29); HRESIMS *m/z* 361.23507 $[M + Na]^+$ ($C_{20}H_{34}O_4Na$ requires 361.23548).

3,4-Dihydroxyclerodan-13*Z*-en-15-oic acid (5): yellow oil; t_R 18.8 min; $[\alpha]_D^{23}$ −2.6 (*c* 1.8, $CHCl_3$); UV (MeCN) λ_{max} (log ϵ) 225 (3.38) nm; ^{13}C and ^1H NMR data, see Tables 1 and 2, respectively; ESIMS *m/z* 361 $[M + Na]^+$; EIMS *m/z* 338 $[M]^+$ (0.01), 320 (13), 303 (53), 277 (5), 225 (16), 207 (46), 189 (61), 163 (26), 149 (42), 137 (41), 123 (76), 113 (76), 109 (56), 95 (100), 84 (26), 69 (56), 55 (51).

Copalic acid [5-(2-methylene-5,5,8a-trimethyl-(1*R*,4a*R*,8a*R*)-decahydronaphthalen-1-yl)-3-methylpent-2*E*-enoic acid] (6): white amorphous powder; t_R 25.8 min; $[\alpha]_D^{23}$ −14.7 (*c* 1.7, $CHCl_3$); UV (MeCN) λ_{max} (log ϵ) 225 (3.23) nm; ^{13}C and ^1H NMR data, see Tables 1 and 2, respectively; EIMS *m/z* 304 $[M]^+$ (20), 289 (85), 256 (45), 205 (29), 177 (44), 149 (41), 137 (100), 123 (54), 109 (62), 95 (76), 81 (69), 69 (73), 55 (70); HRESIMS *m/z* 327.22953 $[M + Na]^+$ ($C_{20}H_{32}O_2Na$ requires 327.23000).

X-ray Crystal Structure of 5α,8α(2-Oxokolavenic acid) (2).[18] A colorless needlelike crystal was obtained by slow evaporation of an AcOEt−hexane solution. Crystal data: Empirical formula = $C_{20}H_{30}O_3$, M_r = 318.4, T = 140(2) K, crystal system = orthorhombic, space group = $P2_12_12_1$, unit cell dimensions: a = 7.739(2) Å, b = 10.156(2) Å, c = 22.793(4) Å, V = 1791.5(7) Å^3, Z = 4, D_{calc} = 1.181 Mg/m^3, λ = 0.71073 Å, μ(Mo Kα) = 0.077 mm^{-1}, F_{000} = 696, crystal size = 0.23 × 0.10 × 0.08 mm, theta range for data collection: θ = 3.20−25.05°, index ranges = $-8 \le h \le 8$, $-12 \le k \le 12$, $-27 \le l \le 27$, reflections collected = 10 832, independent reflections = 1755 (R_{int} = 0.182, poorly diffracting crystal), completeness to theta = 25.05°, 95.5%, no absorption or extinction corrections were applied. Refinement method: full-matrix least-squares on F^2, data/restraints/parameters = 1755/1/216, S = 1.02, final R indices [$I > 2\sigma(I)$]: R1 = 0.0774, wR2 = 0.1849, R indices (all data): R1 = 0.1075, wR2 = 0.2083, absolute configuration not determined (Friedel pairs merged, $\Delta f''$ set to zero), largest difference peak and hole = 0.26 and −0.25 e Å^{-3}.

X-ray Crystal Structure of 3,4-Dihydroxyclerodan-13*E*-en-15-oic acid (4).[18] A colorless needlelike crystal was obtained by slow evaporation of an AcOEt−hexane solution. Crystal data: Empirical formula = $C_{20}H_{34}O_4$, M_r = 338.47, T = 140(2) K, crystal system = orthorhombic, space group = $P2_12_12_1$, unit cell dimensions: a = 7.2148(4) Å, b = 9.5841(9) Å, c = 27.361(2) Å, V = 1891.9(2) Å^3, Z = 4, D_{calc} = 1.188 Mg/m^3, λ = 0.71073 Å, μ(Mo Kα) = 0.081 mm^{-1}, F_{000} = 744, crystal size = 0.27 × 0.10 × 0.07 mm, theta range for data collection: θ = 3.20−25.05°, index ranges = $-8 \le h \le 8$, $-12 \le k \le 12$, $-27 \le l \le 27$, reflections collected = 11 611, independent reflections = 1866 (R_{int} = 0.051), completeness to theta = 25.02°, 95.1%, no absorption or extinction corrections were applied. Refinement method: full-matrix least-squares on F^2, data/restraints/parameters = 1866/3/231, S = 0.97, final R indices [$I > 2\sigma(I)$]: R1 = 0.0328, wR2 = 0.0570, R indices (all data): R1 = 0.0498, wR2 = 0.9614, absolute configuration not determined (Friedel pairs merged, $\Delta f''$ set to zero), largest difference peak and hole = 0.15 and −0.12 e Å^{-3}.

Antifungal Assays. Extract, fractions, and pure compounds were tested for their antifungal activity against *Cladosporium cucumerinum* by bioautography on thin-layer chromatograms.[19] The plant extract (100 μg), fractions (100 μg), and pure compounds (100 to 1 μg) were applied on aluminum-backed silica gel plates. They were developed in AcOEt−hexane (1:1), thoroughly dried for complete removal of solvent, and sprayed with a conidial suspension of *C. cucumerinum* in a nutritive medium. Clear inhibition zones were observed against a dark gray background after 3 days incubation in polystyrene boxes with a moist atmosphere. Miconazole (Sigma) was used as reference compound.

TLC Bioassay for Inhibition of Acetylcholinesterase. The plant extract (10 μg), fractions (10 μg), and pure compounds (10 to 0.01 μg) were tested for the inhibition of acetylcholinesterase by bioautography on thin-layer chromatograms.[15] After migration in AcOEt−hexane (1:1), the TLC plates were dried for complete removal of solvent. A solution of acetylcholinesterase in 0.05 M Tris-HCl (pH 7.8) was sprayed over the TLC plate. After 20 min incubation at 37 °C in a moist atmosphere, the bioautograms were then sprayed with a 1:4 mixture of solutions of naphthyl acetate (250 mg) in ethanol (100 mL) and Fast Blue salt B (400 mg) in distilled water (160 mL). After 1−2 min, active compounds appeared as clear spots against a purple background. Galanthamine (Sigma) was used as reference compound.

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Supporting Information Available: Crystallographic information of compounds **2** and **4** are available free of charge via the Internet at http://pubs.acs.org.

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(18) Crystallographic data for structures reported in this paper have been deposited with the Cambridge Crystallographic Data Centre and allocated the deposition numbers CCDC 297329 for compound **2** and CCDC 297330 for compound **4**. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0) 1223-336033 or e-mail: deposit@ccds.cam.ac.uk).
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